TRADE SECRET

Study Title

H-28072: Unscheduled DNA Synthesis (UDS) Test with Mammalian Cells *In Vivo*

Testing Guidelines

ICH S2A document April 24, 1996
ICH S2B document November 21, 1997
EC Commission Annex V to Directive 67/548/EEC, Directive 2000/32/EC, B.39
OECD Guideline for the Testing of Chemicals, Guideline 486 (1998)

Authors

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Final Report Date

14 August 2007

Testing Facility

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for
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Newark, DE 19714-0050, U.S.A.

BioReliance Study Number

AC03GE.381.BTL

Work Request No:

17319

Service Code

484

STATEMENT OF COMPLIANCE

Study No. AC03GE.381.BTL was conducted in compliance with the U.S. EPA Good Laboratory Practice Regulations as published in 40 CFR 792, and the OECD Principles of Good Laboratory Practice in all material aspects, with the following exceptions,

> The identity, strength, purity, and composition or other characteristics to define the test substance and the stability of the test substance have not been determined by the testing facility or the Sponsor.
>
> Analyses to determine the uniformity or concentration of the negative control, positive control and test substance mixtures and their stability were not performed by the testing facility or the Sponsor.

Applicant/Sponsor: E. I. du Pont de Nemours and Company
Wilmington, DE 19898, U.S.A.

BioReliance Study Director: Kamala Pant — Kamala Pant, M.S. — 14 Aug 2007 — Date

BioReliance Study Management: Ramadevi Gudi — 14 Aug 2007 — Date

Applicant/Sponsor: _____ — DuPont Representative — _____ — Date

Quality Assurance Statement

Study Title: H-28072: Unscheduled DNA Synthesis (UDS) Test with Mammalian Cells In Vivo

Study Number: AC03GE.381.BTL

Study Director: Kamala Pant, M.S.

Quality Assurance performed the inspections listed below for this study. Verification of the study protocol was also performed and documented by QA. Procedures, documentation, equipment records, etc., were examined in order to assure that the study was performed in accordance with the U.S. EPA GLPs (40 CFR 792) and the OECD Principles of Good Laboratory Practice, and to assure that the study was conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected, and report dates of QA inspections of this study.

Inspect On:	19-Jun-07 - 20-Jun-07 To Study Dir 20-Jun-07 To Mgmt 20-Jun-07
Phase:	Scoring the slides
Inspect On:	10-Jul-07 - 10-Jul-07 To Study Dir 10-Jul-07 To Mgmt 23-Jul-07
Phase:	Draft Report and Data Audit
Inspect On:	13-Aug-07 - 13-Aug-07 To Study Dir 03-Aug-07 To Mgmt 14-Aug-07
Phase:	Draft to Final Report

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Michael Tekin, B.S.
QUALITY ASSURANCE

14 Aug 2007
DATE

CERTIFICATION

We, the undersigned, declare that this report provides an accurate evaluation of data obtained from this study.

Issued by Study Director: Kamala Pant 14 Aug 2007

Kamala Pant, M.S.
BioReliance, U.S.A.

Date

Approved by
Study Sponsor:

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STUDY INFORMATION

Substance Tested:
- HFPO Dimer Acid Ammonium Salt
- 2,3,3,3-tetrafluoro-2-(heptafluoropropozy)propionic acid, ammonium salt
- 62037-80-3 (CAS Number)

Haskell Number: H-28072

Composition: 82.6% Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy)propionate*
13.9% Water
3.5% Ammonium
0.41% Organic Impurities

*Note: The Ammonium-2,3,3,3-tetrafluoro-2-(heptafluoropropoxy) propionate component (HFPO Dimer ammonium salt) contains 0.1 ppm HFPO trimer ammonium salt.

Purity: See composition, above

Physical Characteristics: Clear and colorless concentrated aqueous solution

Stability: The test substance appeared to be stable under the conditions of the study; no evidence of instability was observed.

Study Initiated/Completed: 16 May 2007 / (see report cover page)

Experimental Start/Termination: 29 May 2007 / 26 June 2007

SUMMARY

The test substance, H-28072, was tested in the Unscheduled DNA Synthesis (UDS) Test in Mammalian Cells *In Vivo*. The UDS assay was used to evaluate the potential of the test substance to induce unscheduled DNA synthesis in primary cultures of hepatocytes of test substance exposed male rats.

For the Pilot Toxicity Assay, the test substance was administered via oral gavage to male rats at 1, 10, 100, 1000 and 2000 mg/kg body weight (bw) in a total volume of 10 mL/kg bw. All animals appeared normal less than four hours following dosing, and 1, 2 and 3 days following dosing except for two of five 2000 mg/kg animals that were observed to be lethargic only on Day 1. Individual body weights and clinical sign observations are summarized in Table 1.

Based on the information obtained from the Pilot Toxicity Assay, the high dose for the UDS assay was set at the maximum tolerated dose which was 2000 mg/kg bw, accompanied by two lower doses of 500 and 1000 mg/kg bw.

In the UDS test, H-28072, was administered to 5 male rats per dose for 2 to 4 hours and 12 to 16 hours at doses of 500, 1000 and 2000 mg/kg body weight (bw). Two additional groups of 5 rats each, for each timepoint, received a single oral dose of distilled water or 35 mg/kg dimethylnitrosamine (DMN) which served as the vehicle and positive control, respectively. The vehicle control, positive control and test substance were administered at a constant volume of 10 mL/kg bw by a single oral gavage injection. One male rat from the 12-16 hour treatment at 2000 mg/kg bw was observed to be normal less than four hours after dose administration and displayed lethargy and piloerection at the time of harvest. No mortality or clinical signs were observed in any of the remaining test substance- or control-treated harvested animals immediately following dosing or prior to harvest and are summarized in Table 2.

The group mean net nuclear grain (NG) counts for animals treated with H-28072, were not increased when compared to the vehicle control. For the 2 to 4 hour time point, the group mean NG counts for the test substance-treated animals were -3.7, -4.3 and -3.9 with ≤4% of cells in repair (cells with ≥ 5 NG) for the 500, 1000 and 2000 mL/kg bw animals, respectively. The group mean NG count for the vehicle control group was -4.2 with 2% of cells in repair. For the 12- to 16-hour time point, the group mean NG counts for the test substance-treated animals were -4.3, -4.0 and -4.8 with ≤2% of cells in repair (cells with ≥ 5 NG) for the 500, 1000 and 2000 mL/kg bw animals, respectively. The group mean NG count for the vehicle control group was -4.3 with 2% of cells in repair. The positive control group mean NG counts were 15.0 and 16.0 for the 2 to 4 hour and 12 to 16 hour, respectively. The percentage of cells in repair for the positive control group was 93% and 95% for the 2 to 4 hour and 12 to 16 hour, respectively. Results are summarized in Tables 3 and 4.

The test substance, H-28072, did not induce a significant increase in the mean number of net nuclear grain counts (i.e., an increase of at least 5 counts over the vehicle control group) in hepatocytes isolated either 2 to 4 hours or 12 to 16 hours after dose administration. H-28072, was concluded to be negative in the Unscheduled DNA Synthesis (UDS) Test in Mammalian Cells *In Vivo*.

PURPOSE

The purpose of this study was to evaluate the potential of H-28072 to induce unscheduled DNA synthesis (UDS) in primary cultures of hepatocytes obtained from H-28072-treated rats. A copy of the study protocol is included in Appendix A.

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test substance, H-28072, was received by BioReliance on 16 May 2007 and was assigned the code number AC03GE. The test substance was described by the Sponsor as a liquid, which should be stored at room temperature with no expiration date provided. Upon receipt, the test substance was described as a colorless liquid, and was stored at room temperature and protected from exposure to light.

The substance used as the vehicle control was distilled water (CAS #7732-18-5, Lot #1347930, expiration date July 2008) obtained from Gibco.

Dimethylnitrosamine (DMN, CAS# 62-75-9, Lot #FIN01, assigned expiration date of 09 January 2009) was obtained from TCI America and was diluted in distilled water (Gibco, Lot #1347930) to a stock concentration of 3.5 mg/mL for use as the positive control.

The dosing preparation of the positive control agent, dimethylnitrosamine (DMN), was not analyzed due to safety concerns. However, the vehicle and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the vehicle and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test. Historical control data are presented in Appendix B.

MATERIALS AND METHODS

Test System

Male Sprague-Dawley rats were obtained from Harlan Sprague Dawley, Inc., Frederick, MD. Receipt dates, age and body weight ranges for the animals are indicated in the following table. Individual body weights are reported in Tables 1 and 2.

Assay Type	Date Received	at Randomization	
		Age	Body Weight Range (g)
Pilot Toxicity Assay	22 May 2007	9 weeks 4 days	284.6 to 304.0
UDS Assay	29 May 2007	9 weeks 3 days	256.4 to 275.2

Animal Welfare Provisions

The number of animals, animal procedures and experimental design used in the Unscheduled DNA Synthesis (UDS) Test were reviewed and approved by the BioReliance Institutional Animal Care and Use Committee #62. All procedures involving animals were performed by specifications recommended in The Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996).

Animal Receipt and Quarantine

Animals were obtained from a source monitored for evidence of adventitious agents and were quarantined for no less than 5 days prior to dose administration. The rats were observed each working day for signs of illness, unusual food and water consumption, and other conditions of poor health. The animals were judged to be healthy prior to utilization in the assay.

Animal Care

Animals were housed in an AAALAC-accredited facility with a controlled environment of 50 ± 20% relative humidity and 72 ± 3°F temperature with a 12 hour light/dark cycle. The animal rooms were supplied with at least 10 changes of fresh HEPA-filtered air every hour. Rats of the same sex were housed up to five per rodent Micro-Barrier cage. Cages were placed in the racks equipped with Micro-VENT full ventilation, HEPA filtered system. The purpose of this system is to supply uninterrupted positive air to each individual rodent Micro-Barrier cage and to capture the effluent air from each cage and re-filter the air (HEPA) prior to introducing the air back into the room. Heat-treated hardwood chips were used for bedding. Animals had free access to tap water and a certified laboratory rodent chow, which has been analyzed for environmental contaminants.

Test Substance Solubility Determination

A solubility/workability evaluation was performed on the test substance to determine which vehicle would produce the maximum soluble concentration or workable suspension for use in the study. Distilled water was the only vehicle evaluated when the test substance was observed to be soluble at 200 mg/mL, the highest concentration required to yield the maximum dose level of 2 g/kg bw when dosed to animals via oral gavage at a dosing volume of 10 mL/kg bw.

Dose Selection

Dose selection for the UDS assay was determined based on the results of the Pilot Toxicity Assay in which the test substance was administered as a single oral gavage at doses of 1, 10

100, 1000 and 2000 mg/kg bw at a volume of 10mL/kg bw. The animals were assigned to four experimental groups of two males and one group of five males (for the highest concentration) each according to a computer-generated program which is based on distribution according to body weight. Each animal was given a sequential number and identified by ear tag. Clinical signs were observed after dose administration and each day thereafter for 3 days with body weights taken on Days 1 and 3. After the completion of the Pilot Toxicity Assay the dose levels selected for use in the UDS Assay were 500, 1000 and 2000 mg/kg bw administered via a single oral gavage which is an acceptable method of test substance concentrations to laboratory animals. Negative and positive controls were administered by the same route and frequency as the test substance.

UDS Assay

The UDS assay was conducted using established and validated procedures (Butterworth, 1987). The animals were assigned to ten experimental groups of five males each according to a computer-generated program which is based on distribution according to body weight. Each animal was given a sequential number and identified by ear tag. The study design was as in the following table:

	Number of Rats Dosed	Number of Rats Used for Hepatocyte Cultures	
		2 to 4 hour	12 to 16 hour
Vehicle Control (distilled water)	10	3	3
500 mg/kg bw	10	3	3
1000 mg/kg bw	10	3	3
2000 mg/kg bw	10	3	3
DMN 35 mg/kg bw	10	3	3

Dose Preparation and Administration

The test substance-vehicle mixture, the vehicle control and positive control were administered via a single oral gavage at a dosing volume of 10 mL/kg bw. All rats in the experimental and control groups were weighed prior to dose administration and the dose volume was based on individual body weights. To ensure that three dosed animals would be available per group for UDS evaluation, five animals were dosed in each group. Animals were observed after dose administration and prior to harvest for clinical signs of chemical effect.

Preparation of Hepatocyte Cultures

The methods used for isolation and culturing of hepatocytes are modifications of the procedures used by Williams (1976 and 1979). For preparation of hepatocyte cultures, each rat was anesthetized by inhalation of isoflurane and a midventral incision was made to expose the liver. The liver was perfused with 0.5 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) solution followed by collagenase solution (80-100 units Type I collagenase/mL culture medium). The liver was removed, transected, and shaken in a dilute

collagenase solution to release the hepatocytes. The cells were pelleted by centrifugation, resuspended in complete Williams' medium E (WME; buffered with 0.01 M HEPES, supplemented with 2 mM L-glutamine, 50 µg/mL gentamicin and 10% fetal bovine serum).

Approximately 5 x 10^5 cells were seeded into each of six 35mm tissue culture dishes containing 25mm coverslips and preconditioned complete WME (i.e., complete WME medium in 35mm tissue culture dishes incubated overnight in a humidified atmosphere of 5±1% CO_2 and 37±1°C). A minimum of 6 cultures were set up for each rat. The hepatocyte cultures were maintained in a humidified atmosphere of 5±1% CO_2 and 37±1°C.

Ninety to 180 minutes after plating, the cells were washed once with complete WME and refed with serum-free WME containing 10 µCi ^{3}H-thymidine/mL. Four hours later, the radioactive medium was removed, the cultures were washed three times in serum-free WME containing 0.25 mM thymidine, and then refed with serum-free WME containing 0.25 mM thymidine and incubated for 17-20 hours.

Seventeen to twenty hours after exposure to thymidine, the coverslips bearing cultures were washed once in serum-free WME. The nuclei were swelled in 1% sodium citrate solution and the cultures fixed in three changes of ethanol-glacial acetic acid fixative (3:1, v/v). The coverslips were allowed to air dry for at least 1 hour before mounting cell side up on glass slides. The slides were labeled with the study number and a code to identify the animal number.

At least three of the six slides for each rat were dipped in photographic emulsion (diluted 1:1 in deionized water) at 43-45°C, allowed to drain and dry for at least 1.5 hours at room temperature and were stored for 7 days at 2-8°C in light tight boxes with a desiccant. Slides were developed in Kodak D-19 developer (diluted 1:1 in deionized water), fixed in Kodak fixer, and stained with hematoxylin-eosin stain.

Scoring

All coded slides were read without knowledge of treatment group. The slides were viewed microscopically under a 100X oil immersion lens. A computer equipped with image analyzing software was interfaced through a video camera with the microscope so that silver grains within each nuclei and the surrounding cytoplasm could be counted. ProtoCOL system Version 3.07 with accompanying support software was used for grain counts. First the number of grains in a nucleus was counted. Then the number of grains in three separate nuclear-size cytoplasmic areas (taken from the area adjacent to the nucleus and which appears to have the highest grain counts) were counted. The counts were captured directly by the software and stored as raw data in an electronic data file created by the software. Fifty nuclei were scored from each of three replicate cultures for a total of 150 nuclei from each rat. Replicative DNA synthesis is evidenced by nuclei completely blackened with grains, and such cells were not counted. Cells

exhibiting toxic effects of treatments, such as irregularly shaped or very darkly stained nuclei, were not counted.

Presentation of Data

A net nuclear grain count was calculated for each nucleus scored by subtracting the mean cytoplasmic area count from the nuclear area count. For each treatment slide, the net nuclear counts were averaged and the mean ± standard deviation (S.D.) reported. Also reported are the average mean and S.D. for each animal as well as the percent of cells in repair (cells with ≥ 5 net nuclear grains).

Evaluation of Test Results

All conclusions are based on sound scientific judgment; however, the following is offered as a guide to interpretation of the data. Any mean net nuclear count which was increased by at least five counts over the vehicle control was considered significant (Butterworth *et al.*, 1987). The test substance was judged positive if it induced a dose-related increase with no less than one dose significantly elevated above the vehicle control. A significant increase in the mean net nuclear grain count in at least two successive doses in the absence of a dose response was considered positive. A significant increase in the mean net nuclear grain count at the high dose group only with no evidence of a dose response was considered suspect. A significant increase in the mean net nuclear grain count at one dose with no evidence of a dose response was judged to be equivocal. The test substance was considered negative if no significant increase in the mean net nuclear grain counts was observed. These data may also be used by the Study Director in making a final evaluation of the activity of the test substance.

Criteria for a Valid Test

The proportion of cells in repair in the vehicle control group must be less than 15% and the mean net nuclear grain count of the vehicle control must be less than one. The mean net nuclear grain count of the positive control group must be at least 5 counts over that of the vehicle control group.

Automated Data Collection Systems

The primary computer applications used for the collection of data included:

- Protocol System, Version 3.07
- Kaye Lab Watch Monitoring System (GE Kaye Version 1F1X 3.0)
- LIMS Labware Version 5, Configured Version 1.0.3 (BioReliance)
- MiniTab (Bobstudy GT15.MTB Version 1.1)
- Excel 2003 (Microsoft Corporation)

Records and Archives

All raw data, the protocol, and a copy of all reports will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance Regulatory Affairs/Quality Assurance Unit headquartered at BioReliance 14920 Broschart Road, Rockville, MD 20850. Paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of ten years.

Deviations

No protocol deviations occurred during the execution of the study.

RESULTS AND DISCUSSION

Test Substance Solubility Determination

A solubility/workability evaluation was performed on the test substance to determine which vehicle would produce the maximum soluble concentration or workable suspension for use in the study. Distilled water was the only vehicle evaluated when the test substance was observed to be soluble at 200 mg/mL, the highest concentration required to yield the maximum dose level of 2 g/kg bw when dosed to animals via oral gavage at a dosing volume of 10 mL/kg bw.

Pilot Toxicity Assay

In the Pilot Toxicity Assay, the test substance was administered via oral gavage to male rats at 1, 10, 100, 1000 and 2000 mg/kg body weight (bw) in a total volume of 10 mL/kg bw. All animals appeared normal less than four hours following dosing, and 1, 2 and 3 days following dosing except for two of five 2000 mg/kg-treated animals that were observed to be lethargic only on Day 1. Individual body weights and clinical sign observations are summarized in Table 1.

Based on the information obtained from the Pilot Toxicity Assay, the high dose for the UDS assay was set at the maximum tolerated dose which was 2000 mg/kg bw, accompanied by two lower doses of 500 and 1000 mg/kg bw.

In Vivo UDS Assay

In the UDS test, H-28072, was administered to 5 male rats per dose for 2 to 4 hours and 12 to 16 hours at doses of 500, 1000 and 2000 mg/kg body weight (bw). Two additional groups of 5 rats each, for each timepoint, received a single oral dose of distilled water or 35 mg/kg dimethylnitrosamine (DMN) which served as the vehicle and positive control, respectively. The vehicle control, positive control and test substance were administered at a constant volume of 10 mL/kg bw by a single oral gavage injection. One male rat from the 12-16 hour treatment at 2000 mg/kg bw was observed to be normal less than four hours after dose administration and displayed lethargy and piloerection at the time of harvest. No mortality or clinical signs were observed in any of the remaining test substance- or control-treated harvested animals immediately following dosing or prior to harvest and are summarized in Table 2.

The results of the UDS assay using primary hepatocytes isolated 2 to 4 hours post-exposure are summarized in Table 3. The mean net nuclear grain count for the vehicle control group was -4.2 with 2% of cells in repair. The means of the net nuclear grain counts for the 500, 1000 and 2000 mg/kg bw treatment groups were -3.7, -4.3 and -3.9 with $\leq$4% of cells in repair, respectively. The mean net nuclear grain count for the positive control group was 15.0 with 93% of cells in repair. The mean net nuclear grain counts from the positive control or test

substance-treatment groups were compared to the mean net nuclear grain counts from the vehicle control group. None of the test substance doses caused a significant increase in the mean net nuclear counts. The positive control compound, DMN, at 35 mg/kg bw, induced an increase in the average mean net nuclear grain counts of +19.2 over that of the vehicle control. According to the criteria set for evaluating the test results, the induced increase was considered to be significant in the DMN-treated animals.

The results of the UDS assay using primary hepatocytes isolated 12 to 16 hours post-exposure are summarized in Table 4. The mean net nuclear grain count for the vehicle control group was -4.3 with 2% of cells in repair. The means of the net nuclear grain counts for the 500, 1000 and 2000 mg/kg bw treatment groups were -4.3, -4.0 and -4.8 with $\leq$2% of cells in repair, respectively. The mean net nuclear grain count for the positive control group was 16.0 with 95% of cells in repair. The mean net nuclear grain counts from the positive control or test substance-treatment groups were compared to the mean net nuclear grain counts from the vehicle control group. None of the test substance doses caused a significant increase in the mean net nuclear counts. The positive control compound, DMN, at 35 mg/kg bw, induced an increase in the average mean net nuclear grain counts of +20.3 over that of the vehicle control. According to the criteria set for evaluating the test results, the induced increase was considered to be significant in the DMN-treated animals.

CONCLUSION

All criteria for a valid study were met. The positive and negative controls were within the historical control value ranges summarized in Appendix B. The results of this assay indicate that, under the test conditions, H-28072, did not induce a significant increase in the mean number of net nuclear grain counts in hepatocytes isolated from test substance-treated animals, and was concluded to be negative in the Unscheduled DNA Synthesis (UDS) Test in Mammalian Cells *In Vivo* with H-28072 .

REFERENCES

Butterworth, B.E., Ashby, J., Bermudez, E., Casciano, D., Mirsalis, J., Probst, G. and Williams, G. A protocol and guide for the in vivo rat hepatocyte DNA-repair assay. Mutation Research, 189:123-133, 1987.

OECD Guideline 486, Unscheduled DNA Synthesis (UDS) Test with Mammalian Cells *In Vivo*, Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

San, R.H.C., Sly, J.E. and Raabe, H.A. (1996) Unscheduled DNA synthesis in rat hepatocytes following *in vivo* administration of dimethylnitrosamine via different routes. Environ. Molec. Mutagen. 27, Suppl. 27: 58.

Williams, G.M., (1976) Carcinogen-induced DNA repair in primary rat liver cell cultures, a possible screen for chemical-carcinogens. Cancer Letters, 1:231-236.

Williams, G.M., (1979) The detection of chemical mutagens/carcinogens by DNA repair and mutagenesis in liver cultures. In: Chemical Mutagens, Vol. VI, F.J. DeSerres and A. Hollaender, eds. Plenum Press, New York, pp 61-79.

European Commission Annex V to Directive 67/548/EEC, Directive 2000/32/EC, B.39. Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*.

OPPTS Guidelines 870.5550 Unscheduled DNA Synthesis in Mammalian Cells in Culture, EPA712-C-96-230 June 1996.

Table 1
Clinical Signs and Body Weights for Pilot Toxicity Test with H-28072
(BioReliance Study No. AC03GE.381.BTL)

Group	Animal Number	Body Weight at Dosing (g)	Observation at Dosing	Day 1 Body Weight (g)	Body Weight Difference from Dosing (g)	Day 1 Observations	Day 2 Observations	Day 3 Body Weight (g)	Body Weight Difference from Day 1 (g)	Day 3 Observations
1.0 mg/kg	151	289.6	N	293.4	3.8	N	N	294.4	1.0	N
	152	297.6	N	295.6	-2.0	N	N	303.3	7.7	N
10 mg/kg	153	284.6	N	281.7	-2.9	N	N	293.6	11.9	N
	154	304.0	N	305.2	1.2	N	N	318.9	13.7	N
100 mg/kg	155	287.3	N	286.3	-1.0	N	N	297.0	10.7	N
	156	300.8	N	295.3	-5.5	N	N	307.8	12.5	N
1000 mg/kg	157	291.4	N	284.2	-7.2	N	N	301.3	17.1	N
	158	298.8	N	295.3	-3.5	N	N	311.3	16.0	N
2000 mg/kg	159	297.0	N	279.8	-17.2	N	N	296.2	16.4	N
	160	298.6	N	284.0	-14.6	N	N	302.6	18.6	N
	161	294.4	N	284.1	-10.3	N	N	290.0	5.9	N
	162	298.9	N	266.9	-32.0	L	N	283.2	16.3	N
	163	287.9	N	258.2	-29.7	L	N	275.7	17.5	N

N = Normal
L = Lethargy

Table 2
Clinical Signs and Body Weights for UDS Test with H-28072
Male Sprague Dawley Rats
(BioReliance Study No. AC03GE.381.BTL B1)

12 to 16 hour exposure					2 to 4 hour exposure			
Group	Animal Number	Body Weight at Dosing (g)	<4 Hours	at Harvest	Animal Number	Body Weight at Dosing (g)	<4 Hours	at Harvest
Distilled Water	301	256.4	N	N	326	277.5	N	N
	302	261.0	N	N	327	285.3	N	N
	303	264.6	N	N	328	289.2	N	N
500 mg/kg	306	259.6	N	N	331	279.6	N	N
	307	263.2	N	N	332	271.2	N	N
	308	263.7	N	N	333	279.5	N	N
1000 mg/kg	311	259.2	N	N	336	278.0	N	N
	312	263.3	N	N	337	282.4	N	N
	313	264.3	N	N	338	283.4	N	N
2000 mg/kg	316	257.9	N	N	341	278.1	N	N
	317	262.6	N	L, P	342	279.7	N	N
	318	263.8	N	N	343	271.5	N	N
35 mg/kg DMN	321	258.2	N	N	346	279.9	N	N
	322	263.5	N	N	347	279.9	N	N
	323	265.7	N	N	348	281.9	N	N

N = Normal
L = Lethargy
P = Piloerection
Observations are for animals harvested and evaluated for UDS. Extra dosed animals observations are not recorded.

TABLE 3
Summary of UDS Assay with H-28072
2 to 4 Hour Exposure
AC03GE.381.BTL.B1

Group	Ear Tag ID.	Slide Code	Cells Scored	per Animal: Mean Grain Counts ± S.D.[1] Nuclear	Cytoplasmic	Net per Nucleus	Cells in Repair		per Treatment Group: Mean Net ± S.D.#[2]	Cells in Repair
Distilled Water (10 mL/kg)										
	326	18	150	7.8 ± 3.7	10.5 ± 3.7	-2.6 ± 3.7	3%			
Vehicle	327	11	150	6.6 ± 3.3	12.1 ± 4.8	-5.5 ± 4.4	1%		-4.2 ± 1.5	2%
	328	16	150	6.3 ± 3.0	10.7 ± 3.1	-4.3 ± 3.3	1%			
H-28072 (mg/kg)										
	331	32	150	8.8 ± 4.4	12.8 ± 5.2	-4.0 ± 4.3	1%			
500	332	21	150	7.7 ± 4.5	11.4 ± 4.8	-3.7 ± 4.5	5%		-3.7 ± 0.2	4%
	333	20	150	8.8 ± 4.3	12.3 ± 4.8	-3.5 ± 4.4	4%			
	336	33	150	9.9 ± 4.9	15.8 ± 4.9	-6.0 ± 4.3	2%			
1000	337	14	150	9.4 ± 4.2	13.3 ± 4.7	-3.9 ± 3.9	3%		-4.3 ± 1.5	2%
	338	34	150	8.3 ± 4.0	11.3 ± 4.4	-3.0 ± 3.4	3%			
	341	38	150	10.8 ± 4.4	15.9 ± 5.5	-5.1 ± 4.7	1%			
2000	342	26	150	10.8 ± 4.5	15.7 ± 5.6	-4.9 ± 4.7	1%		-3.9 ± 2.0	1%
	343	30	150	6.4 ± 3.3	8.0 ± 3.3	-1.6 ± 2.9	1%			
Positive Control: Dimethylnitrosamine (mg/kg)										
	346	27	150	19.9 ± 7.4	5.8 ± 2.1	14.2 ± 6.9	93%			
35	347	24	150	27.9 ± 9.7	12.1 ± 6.9	15.8 ± 7.8	95%	*	15.0 ± 0.8	93%
	348	17	150	24.5 ± 9.4	9.4 ± 5.8	15.1 ± 7.8	92%			

[1] Standard deviation reflecting slide to slide variation

[2] S.D.#: Standard deviation reflecting variation between animals

* Significant (see protocol Section 9.0, Evaluation of Test Results)

TABLE 4
Summary of UDS Assay with H-28072
12 to 16 Hour Exposure
AC03GE.381.BTL.B1

Group	Ear Tag ID.	Slide Code	Cells Scored	per Animal: Mean Grain Counts ± S.D.[1] Nuclear	Cytoplasmic	Net per Nucleus	Cells in Repair		per Treatment Group: Mean Net ± S.D.#[2]	Cells in Repair
Distilled Water (10 mL/kg)										
	301	15	150	9.0 ± 4.8	13.1 ± 4.7	-4.0 ± 4.6	4%			
Vehicle	302	39	150	7.9 ± 3.9	13.0 ± 4.6	-5.1 ± 4.3	2%		-4.3 ± 0.7	2%
	303	40	150	7.1 ± 3.4	10.9 ± 4.0	-3.8 ± 3.3	0%			
H-28072 (mg/kg)										
	306	12	150	7.4 ± 3.4	11.5 ± 3.7	-4.1 ± 3.2	0%			
500	307	37	150	9.5 ± 5.9	13.4 ± 5.9	-3.9 ± 5.5	2%		-4.3 ± 0.6	1%
	308	19	150	9.2 ± 4.0	14.3 ± 4.5	-5.1 ± 4.0	0%			
	311	29	150	9.4 ± 4.3	14.4 ± 4.9	-5.0 ± 4.5	2%			
1000	312	25	150	8.3 ± 3.6	12.1 ± 4.1	-3.8 ± 3.5	1%		-4.0 ± 0.9	2%
	313	35	150	7.7 ± 5.0	10.9 ± 4.9	-3.2 ± 4.5	4%			
	316	22	150	6.6 ± 3.5	10.1 ± 4.6	-3.5 ± 3.6	2%			
2000	317	36	150	9.3 ± 4.1	14.6 ± 5.3	-5.3 ± 3.9	1%		-4.8 ± 1.2	1%
	318	13	150	9.8 ± 4.3	15.5 ± 5.4	-5.7 ± 4.4	1%			
Positive Control: Dimethylnitrosamine (mg/kg)										
	321	31	150	22.8 ± 13.0	6.7 ± 3.6	16.1 ± 11.5	95%			
35	322	28	150	25.6 ± 11.7	8.4 ± 4.4	17.2 ± 10.9	95%	*	16.0 ± 1.2	95%
	323	23	150	20.1 ± 8.9	5.3 ± 2.6	14.8 ± 8.0	95%			

[1] Standard deviation reflecting slide to slide variation

[2] S.D.#: Standard deviation reflecting variation between animals

* Significant (see protocol Section 9.0, Evaluation of Test Results)

APPENDIX A

Study Protocol

DuPont-23219
BioReliance Study Number: AC03GE.381.BTL

Unscheduled DNA Synthesis (UDS) Test
with Mammalian Cells *In Vivo*

1.0 PURPOSE

The purpose of this study is to evaluate the potential of the test substance to induce unscheduled DNA synthesis (UDS) in primary cultures of hepatocytes obtained from test substance-treated rats.

2.1	Sponsor Name:	E.I DuPont de Nemours and Company
2.2	Address:	Dupont Haskell Laboratory P.O. Box, 50, 1090 Elkton Road Newark, DE 19714-0050
2.3	Representative:	E. Maria Donner, Ph.D. Phone: 302-366-5251 Fax: 302-366-5207 Email: Maria.Donner@usa.dupont.com
2.4	Work Request No.:	17319
2.5	Haskell Number:	H-28072
2.6	Service Code :	484
2.4	Sponsor Project No:	23219

3.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

3.1	Test Substance:	H-28072
	Storage Temperature:	Ambient, 2 to 8°C or -5 to -40°C based on the shipping conditions of ambient, cool packs or dry ice, respectively.
	Storage Parameters:	Unless otherwise indicated, all test substances will be stored in the dark and solids will be stored with desiccant.
	Purity:	84.5%, an adjustment for purity or active ingredient will be made using a correction factor of 1.18.
	Molecular Weight:	347.09

3.2 Controls: Negative: Test substance vehicle
Positive: Dimethylnitrosamine (DMN)

3.3 Characterization and Stability of the Test Substance and Test Substance Mixtures

The Sponsor will be directly responsible for determination and documentation of the analytical purity, composition and stability of the test substance, and the stability and strength of the test substance in the solvent (or vehicle).

Test substance dosing solutions will not be analyzed.

No analyses will be performed on the positive control substance, which will be characterized by the Certificate of Analysis maintained by BioReliance.

3.4 Test Substance Retention Sample

Since the in-life portion of this study is less than four weeks in duration, BioReliance will not retain a reserve sample of the test substance.

3.5 Residual Test Substance and Dosing Preparations

Dosing preparations, excluding those saved for concentration or homogeneity analysis, will be disposed of following administration to the test system. Following finalization of the report, residual test substance will be discarded unless otherwise indicated by the Sponsor.

4.0 TESTING FACILITY AND KEY PERSONNEL

4.1 Name: Toxicology Testing Facility
BioReliance

4.2 Address: 9630 Medical Center Drive
Rockville, MD 20850

4.3 Study Director: Kamala Pant, M.S.
Phone: 301-610-2192
Fax: 301-738-2362
E-mail: Kamala.Pant@bioreliance.com

5.0 TEST SCHEDULE

5.1 Proposed Experimental Initiation Date: 29 May 2007

5.2 Proposed Experimental Completion Date: 02 July 2007

5.3 Proposed Report Date: 23 Jul 2007

6.0 TEST SYSTEM

Rodents are acceptable models for mutagenicity and DNA damage studies.

6.1 Source: Harlan Sprague Dawley, Inc.
Frederick, MD (or other approved alternates)

6.2 Gender and Species: male, Sprague-Dawley rats

6.3 Age at experimental initiation: 8 to 12 weeks

6.4 Body weight at experimental initiation: 200 to 400 grams

7.0 EXPERIMENTAL DESIGN AND METHODOLOGY

The experimental design follows that described by Butterworth *et al.* (1987). Hepatocytes will be isolated from male rats at two time points (2-4 hours and 12-16 hours) following the administration of three concentrations of test substance as well as positive and negative controls. The UDS test will be evaluated on the basis of incorporation of tritiated thymidine (^{3}H-TdR) into the hepatocyte DNA, presumably as a consequence of DNA repair. The study design will be as follows:

Treatment	Animals to be Evaluated for UDS after Dose Administration	
	2-4 hours	12-16 hours
Negative Control	3	3
High Test Dose	3	3
Mid Test Dose	3	3
Low Test Dose	3	3
Positive Control (DMN)	3	3

7.1 Selection of Test Substance Vehicle

A solubility determination will be conducted to determine the maximum soluble or workable concentration of the test article in the vehicle. Vehicles compatible with this test system, in order of preference, include, but are not limited to, water (purified or sterile water for injection), saline (0.9% sodium chloride for injection) or phosphate buffered saline (PBS), aqueous solutions of carboxymethylcellulose or methycellulose and corn oil. The vehicle of choice will be the one that allows preparation of dose formulations required to achieve targeted doses.

Selection of the test article vehicle for this study may be based on the results of the solubility test conducted for other toxicology studies being performed at BioReliance.

7.2 Animal Welfare Provisions

This study is not duplicative or unnecessary. The number of animals, animal procedures and experimental design used for this study have been reviewed and were approved by the BioReliance Institutional Animal Care and Use Committee #62. All procedures involving animals performed at BioReliance follow the specifications recommended in The Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996).

7.3 Selection of Test Substance Vehicle

Unless the Sponsor has indicated the test substance vehicle, a solubility determination will be conducted to measure the maximum soluble concentration or workable suspension of test substance in vehicle. Vehicles compatible with this test system, in order of preference, include, but are not limited to, distilled water or saline, 1% carboxymethylcellulose (CMC) in water, and corn oil. The vehicle of choice will be that allowing preparation of dosing solutions/suspensions required to achieve targeted dose levels.

7.4 Dose Selection

Selection of dose levels for the UDS assay will be based on toxicity of the test substance but will not exceed 2 g/kg body weight (bw). In the absence of toxicity data, a pilot study will be performed at a dose level of 2 g/kg bw using five male rats. Three or more lower concentrations will be tested using two male rats each. If dose administration produces no treatment-related mortality, the high dose for the definitive UDS assay will be 2 g/kg bw. In the event of mortality in excess of 50% at 2 g/kg bw in the pilot study, a secondary toxicity study will be performed using at least four test substance dose levels, each containing five male rats. Animals will be observed after dose administration and each working day thereafter for at least 3 days for clinical signs of chemical effect. Body weights will be recorded prior to dose administration and no less than 1 and 3 days after dose administration.

Unless specified otherwise by the Sponsor, the high dose for the UDS assay will be the maximum tolerated dose (to a maximum of 2 g/kg bw), or that which produces some indication of toxicity, such as reduction in body weight gain, clinical signs of pharmacotoxic effect, or mortality. The LD_{50} may be selected for the high dose, provided that a sufficient number of animals are likely to survive to the 16 hour post-exposure harvest. Two additional dose levels will be tested, approximately one-half and one-fourth of the high dose.

7.5 Route and Frequency of Administration

The test substance will be administered via oral gavage as a single administration (or by another route if deemed more appropriate by the Sponsor). Oral gavage is an acceptable method for administration of test substance concentrations to laboratory

animals. The negative and positive controls will be administered by the same route as that for the test substance.

7.6 Controls

7.6.1 Negative control

The test substance vehicle will be used as the negative control.

7.6.2 Positive control

Dimethylnitrosamine (DMN) at a dose level of 35 mg/kg bw will be the positive control for the 2-4 and 12-16 hour sacrifices (San *et al.*, 1996).

7.7 Animal Receipt and Quarantine

Animals will be obtained from a source monitored for evidence of adventitious agents and will be quarantined for no less than 5 days prior to dose administration. The animals will be observed each working day for signs of illness, unusual food and water consumption, and other general conditions of poor health. All animals will be judged to be healthy prior to utilization in the study.

7.8 Animal Care

Animals will be housed in an AAALAC-accredited facility with a controlled environment of 50 ± 20% relative humidity and 72 ± 3°F with a 12 hour light/dark cycle. The animal rooms will be supplied with at least 10 changes of fresh HEPA-filtered air every hour. Rats of the same sex will be housed up to five per rodent Micro-Barrier cage. Cages will be placed on the racks equipped with an automatic watering system and Micro-VENT full ventilation, HEPA filtered system. The purpose of this system is to supply uninterrupted positive air to each individual rodent Micro-Barrier cage and to capture the effluent air from each cage and re-filter the air (HEPA) prior to introducing the air back into the cage. Heat-treated hardwood chips will be used for bedding. Animals will have free access to tap water and a certified laboratory rodent chow, which has been analyzed for environmental contaminants.

7.9 Randomization

The animals will be assigned to ten groups of five males each using a randomization procedure which is based on equalization of group mean body weights. A minimum of three animals per group will be sacrificed and evaluated for UDS. Additional animals may be designated and dosed as replacement animals in the high dose group to be used in the event of mortality prior to scheduled sacrifice. This will be done at the discretion of the Study Director after evaluation of the toxicity data. Each animal will be given a sequential number and identified by ear tag.

7.10 Dose Preparation and Administration

The test substance-vehicle mixture, the negative control alone and the positive control will be given as a single administration. The rate of administration for the test substance-vehicle mixture and negative control alone will be 10 mL/kg bw unless larger volumes, up to 20 mL/kg bw, are required to deliver the targeted dose. The positive control will be administered at a volume of 10 mL/kg bw. All rats in the experimental groups will be weighed and the dose volume will be based on individual body weight.

7.11 Preparation of Hepatocyte Cultures

The methods used for isolation and culturing of hepatocytes are modifications of the procedures used by Williams (1976 and 1979). For preparation of hepatocyte cultures, each rat will be anesthetized by inhalation of isoflurane and a midventral incision will be made to expose the liver. The liver will be perfused with 0.5 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA) solution followed by collagenase solution (80-100 units Type I collagenase/mL culture medium). The liver will be removed, transected, and shaken in a dilute collagenase solution to release the hepatocytes. The cells will be pelleted by centrifugation, resuspended in complete Williams' medium E (WME; buffered with 0.01 M HEPES, supplemented with 2 mM L-glutamine, 50 µg/mL gentamicin and 10% fetal bovine serum). Approximately 5 x 10^5 cells will be seeded into each of six 35mm tissue culture dishes containing 25mm coverslips and preconditioned complete WME (i.e., complete WME medium in 35mm tissue culture dishes incubated overnight in a humidified atmosphere of 5±1% CO_2 and 37±1°C). A minimum of 6 cultures will be set up for each rat. The hepatocyte cultures will be maintained in a humidified atmosphere of 5±1% CO_2 and 37±1°C.

7.12 UDS Assay

Ninety to 180 minutes after plating, the cells will be washed once with complete WME and refed with serum-free WME containing 10 µCi ^{3}H-TdR/mL. Four hours later, the radioactive medium will be removed, the cultures will be washed three times in serum-free WME containing 0.25 mM thymidine, and then refed with serum-free WME containing 0.25 mM thymidine and incubated for 17-20 hours.

Seventeen to 20 hours after exposure to thymidine, the coverslips bearing cultures will be washed once in serum-free WME. The nuclei will be swelled in 1% sodium citrate solution and the cultures fixed in three changes of ethanol-glacial acetic acid fixative (3:1, v/v). The coverslips will be allowed to air dry for at least 1.5 hours before mounting cell side up on glass slides. The slides will be labeled with the study number and a code to identify the animal number.

At least three of the six slides for each rat will be dipped in Kodak-NTB or equivalent emulsion at 43-45°C, allowed to drain and dry for at least 1.5 hours at room temperature and will be stored for 3-12 days at 2-8°C in light tight boxes with a

desiccant. Slides will be developed in Kodak D-19 developer (diluted 1:1 in deionized H_2O), fixed in Kodak fixer, and stained with hematoxylin-eosin stain.

7.13 Automated Data Collection Systems

The primary computer or electronic system used for the collection of data will include but not be limited to the following:

LIMS, Oracle (Oracle Corporation), Excel 2003 (Microsoft Corporation) and Kaye Lab watch Monitoring System (Kaye, GE)

7.14 Collection of UDS Data

All coded slides will be read without knowledge of treatment group. The slides will be labeled with the study number, study phase and a code number with the slide replicate identification. The slides will be viewed microscopically under a 100X oil immersion lens. An automated colony counter will be interfaced with the microscope so that silver grains within each nuclei and the surrounding cytoplasm can be counted. ProtoCOL System Version 3.07 with accompanying support software will be used for grain counts. If possible, 50 nuclei will be scored from each of three replicate cultures for a total of 150 nuclei from each rat. A minimum of three animals per group will be evaluated for UDS. Replicative DNA synthesis is evidenced by nuclei completely blackened with grains, and will not be counted. Cells exhibiting toxic effects of treatments, such as irregularly shaped or very darkly stained nuclei, will not be counted.

A net nuclear grain count will be calculated for each nucleus scored by subtracting the mean cytoplasmic area count from the nuclear area count. For each rat, as well as for each treatment group, a mean net nuclear grain count and standard deviation (S.D.), as well as the proportion of cells in repair (percentage of nuclei showing ≥ 5 net nuclear grain counts) will be determined.

Unless otherwise indicated, the slides will be discarded after the finalization of the report.

8.0 CRITERIA FOR DETERMINATION OF A VALID TEST

8.1 Negative Control

The proportion of cells in repair in the negative control group must be less than 15% and the mean net nuclear grain count must be less than one.

8.2 Positive Control

The mean net nuclear grain count of the positive control group must be at least 5 counts over that of the negative control group.

Should one of these criteria not be met, the affected portion of the study will be repeated using the appropriate experimental conditions.

9.0 EVALUATION OF TEST RESULTS

9.1 Positive Results

All conclusions will be based on sound scientific judgement; however, the following is offered as a guide to interpretation of the data.

Any mean net nuclear count that is increased by at least five counts over the negative control will be considered significant (Butterworth *et al.*, 1987).

A test substance will be judged positive if it induces a dose-related increase with no less than one dose significantly elevated above the negative control.

A significant increase in the mean net nuclear grain count in at least two successive doses in the absence of a dose response will also be considered positive.

9.2 Equivocal Results

A significant increase in the net nuclear grain count at the high dose only with no evidence of a dose response will be considered suspect.

A significant increase in the net nuclear grain count at one dose level without a dose response will be judged equivocal.

9.3 Negative Results

The test substance will be considered negative if no significant increase in the net nuclear grain counts is observed.

The percentage of cells in repair (cells with ≥5 net nuclear grains) will also be reported. These data may also be used by the Study Director in making a final evaluation of the activity of the test substance.

10.0 REPORT

A report of the results of this study will be prepared by the Testing Laboratory and will accurately describe all methods used for generation and analysis of the data. Results presented will include:

- test substance: identification and CAS no., if known; physical nature and purity, if known; physicochemical properties relevant to the conduct of the study, if known; stability of test substance, if known.
- solvent/vehicle: justification for choice of vehicle; solubility and stability of test substance in solvent/vehicle, if known.

- species and strain of animals used; age and weights of animals;
- number of animals treated and harvested in each treatment group;
- test substance, vehicle, concentrations, and methods of administration used in the assay as well as rationale for their selection;
- treatment and harvest schedule;
- clinical signs of treatment in animals;
- cells used, cell density and number of cell cultures;
- methods used to sacrifice the animals and harvest cells;
- methods used for maintenance of cell cultures including medium, incubation temperature and CO_2 concentration;
- positive and negative controls;
- autoradiographic technique used;
- evaluation of test results and dose-response relationship;
- discussion and interpretation of results;
- historical control values,
- statement of compliance
- quality assurance statement

11.0 RECORDS AND ARCHIVES

All raw data, the protocol, and all reports for procedures performed at BioReliance will be maintained according to Standard Operating Procedure OPQP3040 by the BioReliance RAQA unit headquartered at BioReliance, 14920 Broschart Road, Rockville, MD 20850. Per this SOP, paper records will be retained for at least three years after which time the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials returned to the Sponsor or destroyed will first be copied and the copy will be retained in the BioReliance archives for a minimum of ten years. Raw data, the protocol and reports generated at facilities other than BioReliance will be archived per the contractual arrangements between that facility and the Sponsor.

12.0 REGULATORY REQUIREMENTS/GOOD LABORATORY PRACTICE

This protocol has been written to comply with OECD Guideline 486, Unscheduled DNA Synthesis (UDS) Test with Mammalian Cells *In Vivo*, adopted 21 July 1997, published by OECD, Paris, February 1998.

European Commission Annex V to Directive 67/548/EEC, Directive 2000/32/EC, B.39. Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*.

Health Effects Test Guidelines OPPTS Guidelines 870.5550 Unscheduled DNA Synthesis in Mammalian Cells in Culture.

The study performed at BioReliance will be conducted in compliance with the provisions of the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792 and Part III Toxic Substance Control Act (TSCA) August 1989, and the OECD Principles of Good

Laboratory Practice, as applicable to the product being tested. Multisite activities (if any) will be conducted per SOP ODQP2411.

An in-process phase, the raw data, and report(s) will be inspected per the Standard Operating Procedures (SOPs) of BioReliance by the Quality Assurance Unit of BioReliance for compliance with GLPs, the SOPs of BioReliance and the study protocol. At least one, study-specific, in-process inspection will be performed for this study. A signed QA statement will be included in the final report. This statement will list the study-specific phases inspected, the dates of each inspection, and the dates the results of each inspection were reported to the Study Director and the Study Director's management. In addition, a signed GLP compliance statement will be included in the final report. This statement will cite the GLP guideline(s) with which the study is compliant and any exceptions to this compliance, if applicable, including the omission of characterization or stability analyses of the test substance or its mixtures.

Raw data, the protocol and reports generated at facilities other than BioReliance will be QA audited per the contractual arrangements between that facility and the Sponsor.

Alterations of this protocol may be made as the study progresses. All protocol modifications, rationale for the change(s) and the effective date of the change(s) will be documented, signed, dated and approved by the Study Director, BioReliance QA and the Sponsor. All protocol amendments will be delivered to the Sponsor and all Principal Investigators (if any) via mail, electronic file transfer or fax transmission, as well as internally at the Test Facility, on or as close as possible to the effective date of the amendment.

Deviations from the protocol (i.e., unplanned changes) will be documented in a deviation report or a note to file will be generated. A deviation report will be signed by the Study Director and BioReliance QA. All deviations will be communicated to the Sponsor Representative and, as appropriate to the Principal Investigator(s) and will be identified in the study report.

13.0 REFERENCES

Butterworth, B.E., Ashby, J., Bermudez, E., Casciano, D., Mirsalis, J., Probst, G. and Williams, G. (1987) A protocol and guide for the *in vivo* rat hepatocyte DNA-repair assay. Mutation Research 189:123-133.

OECD Guideline 486, Unscheduled DNA Synthesis (UDS) Test with Mammalian Cells *In Vivo*, Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, published by OECD, Paris, February 1998.

San, R.H.C., Sly, J.E. and Raabe, H.A. (1996) Unscheduled DNA synthesis in rat hepatocytes following *in vivo* administration of dimethylnitrosamine via different routes. Environ. Molec. Mutagen. 27, Suppl. 27: 58.

Williams, G.M. (1976) Carcinogen-induced DNA repair in primary rat liver cell cultures, a possible screen for chemical-carcinogens. Cancer Letters 1:231-236.

Williams, G.M. (1979) The detection of chemical mutagens/carcinogens by DNA repair and mutagenesis in liver cultures. In: Chemical Mutagens, Vol. VI, F.J. de Serres and A. Hollaender, eds. Plenum Press, New York, pp 61-79.

European Commission Annex V to Directive 67/548/EEC, Directive 2000/32/EC, B.39. Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *In Vivo*

OPPTS Guidelines 870.5550 Unscheduled DNA Synthesis in Mammalian Cells in Culture, EPA712-C-96-230 June 1996

14.0 APPROVAL

E. Jean Donner — 16-May-2007
Sponsor Representative — Date

(E. Maria Donner, Ph.D.)

Kamala Paint — 16 May 2007
BioReliance Study Director — Date

Ramadevi Gudi — 16 May 2007
BioReliance Study Management — Date

APPENDIX B

Historical Control Data

In Vivo In Vivo Unscheduled DNA Synthesis (UDS) Assay

Historical Positive and Negative Controls Net Nuclear Grain Counts 2004 - 2006			
Positive Control[a]		Negative Controls[b]	
Mean ± SD	Range	Mean ± SD	Range
2-4 Hour Harvest		2-4 Hour Harvest	
17.4 ± 6.7	8.8 to 35.6	-1.8 ± 1.5	-6.1 to 2.1
12-16 Hour Harvest		12-16 Hour Harvest	
15.4 ± 5.5	5.9 to 27.6	-2.1 ± 1.6	-4.9 to 0.8

[a] Single administration (gavage or intravenous) of dimethylnitrosamine at 35 mg/kg body weight.

[b] Negative controls including but not limited to deionized water, saline, corn oil and carboxymethylcellulose (CMC).

APPENDIX C

Certificate of Analysis

E. I. du Pont de Nemours and Company
Wilmington, DE 19898
USA

CERTIFICATE OF ANALYSIS

This Certificate of Analysis fulfills the requirement for characterization of a test substance prior to a study subject to GLP regulations. It documents the identity and content of the test substance. This work was conducted under EPA Good Laboratory Practice Standards (40 CFR 792).

Haskell Code Number	H-28072
Common Name	HFPO Dimer Acid Ammonium Salt
Purity Percent	82.6%
Other Components	Water – 13.9% Ammonium (excess) – 3.5%
Date of Analysis	July 19, 2007
Recommended reanalysis interval	1 year
Instructions for storage	NRT&H
Reference	DuPont-23285
Analysis performed at	E. I. DuPont de Nemours and Company DuPont Haskell Laboratories Newark, Delaware USA

Peter A. Bloxham, Ph.D.
Analyst's Name

Analyst's signature

23-JULY-2007
Date

Revision #1
July 20, 2007